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DNA Precursor Metabolism and Mitochondrial Genome Stability

ABSTRACT

This project investigated DNA precursor metabolism in mammalian mitochondria, attempting to define relationships between deoxyribonucleoside triphosphate (dNTP) metabolism and mutagenesis in the mitochondrial genome. Specific contributions include: (1) We found that conditions altering the normal balance among the four dNTP pools within the mitochondrion stimulate both point and deletion mutagenesis; (2) dNTP pools in mitochondria from some tissues are highly asymmetric, and this asymmetry contributes toward the elevation of mitochondrial mutation rates compared to nuclear mutation rates; (3) Mitochondrial dNTP pools do not show significant age-related changes in the rat, ruling out such changes as causative agents in aging-related accumulation of mitochondrial mutations; (4) A protein previously identified as a mitochondrial deoxyribonucleotide carrier, responsible for dNTP uptake into mitochondria, plays a quite different metabolic role; (5) Mammalian mitochondria contain a novel ribonucleotide reductase, which may play a significant role in mitochondrial dNTP synthesis.

List of papers submitted or published that acknowledge ARO support during this reporting period. List the papers, including journal references, in the following categories:

(a) Papers published in peer-reviewed journals (N/A for none)

1. Song, S., L. J. Wheeler, and C. K. Mathews (2003) Deoxyribonucleotide pool imbalance stimulates deletions in HeLa cell mitochondrial DNA. J. Biol. Chem. 278, 43893–43896.

2. Song, S., Z. F. Pursell, W. C. Copeland, M. J. Longley, T. A. Kunkel, and C. K. Mathews (2005) DNA precursor asymmetries in mammalian tissue mitochondria and possible contribution to mutagenesis through reduced replication fidelity. Proc. Natl. Acad. Sci. USA 102, 4990–4995.

3. Wheeler, L. J., I. Rajagopal, and C. K. Mathews (2005) Stimulation of mutagenesis by proportional deoxyribonucleotide accuulation in Escherichia coli. DNA Repair 4, 1450–1456.

4. Koc, A., C. K. Mathews, L. J. Wheeler, M. K. Gross, and G. F. Merrill (2006) Thioredoxin is required for deoxyribonucleotide pool maintenance during S phase. J. Biol. Chem. 281, 15058–15063.

5. Mathews, C. K. (2006) DNA precursor metabolism and genomic stability. FASEB J 20, 1300–1314.

6. Lindhurst, M. J., C. Fiermonte, S. Song, E. Struys, F. De Leonardis, P.L. Schwartzberg, A. Chen, A. Castegna, N. Verhoeven, C. K. Mathews, F. Palmieri, and L. G. Biesecker (2006) Knockout of Slc25a19 causes mitochondrial thiamine pyrophosphate depletion, embryonic lethality, CNS malformations, and anemia. Proc. Natl. Acad. Sci. USA 103, 15927–15932.

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1. Song, S., L. J. Wheeler, and C. K. Mathews, Mitochondrial deoxyribonucleotide pools, agents in mitochondrial gene mutations. Presented at “Genetic Toxicology and DNA Repair,” a symposium at Corvallis, Oregon, honoring the memory of Dr. Dale Mosbaugh, October 2004.

2. Song, S., L. J. Wheeler, and C. K. Mathews, Mitochondrial nucleotide metabolism and mutagenesis. Presented at the Gordon Research Conference on Nucleotides, Oligonucleotides, and Nucleic Acids, Newport, Rhode Island, June 2005.

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Shiwei Song	1.00	No
FTE Equivalent:	**1.00**	
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I. Foreword

It has become evident that maintaining genomic stability is crucial to support of normal functions of mitochondria in meeting energy needs of the cell. Accumulation of mitochondrial mutations occurs during normal aging and is correlated with declines in mitochondrial energy generation. In addition, a number of human diseases, which affect the ability of mitochondria to carry out oxidative phosphorylation and other processes in energy generation, result from accumulation of mutations in the mitochondrial genome. Some of these latter conditions result from abnormalities in nucleotide metabolism, which lead to changes in intramitochondrial DNA precursor concentrations. The spontaneous mutation rate for the mitochondrial genome is one to two orders of magnitude higher than that for the nuclear genome. Studies completed during during the grant support period indicate that mammalian mitochondria contain highly asymmetric pools of the four deoxyribonucleoside triphosphates (dNTPs), and that this asymmetry contributes toward the high mutation rate of mitochondrial genes. Most of the work described in this report involves investigations of the metabolic sources of mitochondrial dNTP pools and of the genetic consequences of the high dNTP pool asymmetries that we have discovered in mammalian mitochondria.

II. Table of Contents

III. List of tables

IV. Statement of problem studied

Efficient performance by mitochondria in generating energy through oxidative phosphorylation depends critically upon maintenance of genomic stability within the organelle. As mammals age, mitochondrial efficiency declines, as measured by criteria such as mitochondrial membrane potential and intramitochondrial redox status (1). Also correlated with aging is the accumulation of mutations in mitochondrial DNA, some of which have been identified in organelles from aged humans, but not seen in stored mitochondrial samples taken from the same individuals at earlier stages of their

lives (2, 3, 4). A causal relationship between mitochondrial mutagenesis and loss of function is seen most clearly from studies on several human diseases, in which defined genetic changes lead both to accumulation of mitochondrial mutations and clinical symptoms ascribable to perturbed mitochondrial function (5, 6). Some of these genetic alterations involve enzymes and proteins of DNA precursor metabolism, the principal focus of this project. Our goals are to understand the metabolic sources of the intramitochondrial dNTP pools that supply mitochondrial DNA replication, to learn how the pool sizes are regulated, and to understand how perturbations of normal dNTP metabolism within the mitochondrion contribute toward genomic instability, with consequent effects upon mitochondrial performance.

V. Summary of most important results

Stimulation of mitochondrial mutagenesis by dNTP pool imbalance

Mitochondrial neurogastrointestinal encephalomyopathy (MNGIE), a recessive nuclear gene disorder, affects mitochondrial function and is associated with point mutations and multiple long deletions in mitochondrial DNA of skeletal muscle. Nishino *et al* (7) showed that this condition results from deficiency of thymidine phosphorylase, an enzyme that catalyzes the reversible breakdown of thymidine to thymine plus deoxyribose-1-phosphate. Patients with this condition display elevated levels of circulating thymidine (8), leading to the suggestion that uptake of excess thymidine by mitochondria stimulates salvage synthesis of dTTP, which in turn unbalances the other dNTP pools and stimulates mutagenesis. Working with HeLa cells as a model system (9), we confirmed this prediction by showing that addition of thymidine to the culture medium does cause the mitochondrial dNTP pools to become more imbalanced than the nuclear pools, and that after several months in culture, thymidine-treated cells do accumulate long deletions in their mtDNA.

The pool imbalances that we observed correlate nicely with the pattern of point mutations seen in MNGIE patients by the Hirano laboratory. They reported that the most common point mutations were AT-to-GC transitions where a T in the template strand is followed by two As. dGTP and dTTP both accumulate in our MNGIE model. dGTP couild compete with dATP for incorporation opposite template T, and the elevated dTTP pool would create a next-nucleotide effect, driving formation of the T-A base pairs past the mismatch and preventing its proofreading. With respect to the deletions, various authors have speculated that stalling of DNA polymerase can lead to template-product unwinding at the replication site, and then allowing nascent DNA to pair with a homologous downstream sequence before replication resumes. We don't know whether our pool-imbalanced conditions can cause mitochondrial polymerase to stall, but we note that the thymidine treatment described above caused the mitochondrial dCTP pool to shrink so that it represented only one percent of the total dNTP pool, and this might be a sufficiently low value to limit DNA polymerase activity within the mitochondrion. These observations, I believe, represent the first demonstration that dNTP pool imbalance can be responsible for deletion mutagenesis in living cells and that dNTP pool asymmetries affect mutational pathways in predictable ways.

dNTP asymmetry in mitochondria from mammalian tissues

We completed an analysis of the genetic consequences of the extreme dNTP pool asymmetry we have observed in rat mitochondria from heart and skeletal muscle. Estimated molar concentrations of tissue mitochondria dNTPs are shown in Table I. For comparison, we list data obtained simultaneously for HeLa cell mitochondria. Note that dGTP, which normally comprises 5 to 10 percent of the total dNTP in whole-cell extracts makes up as much as 90 percent of the total in heart and skeletal muscle mitochondria, while the dTTP level is so low that it might well represent a rate-limiting parameter for mtDNA replication.

Table I. Estimated mitochondrial dNTP concentrations in rat tissues

Tissue	Estimated [dNTP], μM				dGTP, % of total
	dATP	dTTP	dCTP	dGTP	
Heart, subsarcolemmal	3.6	0.7	13	110	86
Heart, interfibrillary	4.0	0.8	12	140	89
Liver	3.8	1.3	23	16	36
Brain	11.0	3.4	16	49	62
Skeletal muscle	2.8	0.3	5.3	82	91
HeLa cells	13.4	35.4	7.9	26.8	32

Shown also is our finding that the two classes of heart mitochondria that we isolated have essentially identical mitochondrial dNTP pools. Because our colleague Dr. Tory Hagen had found these two mitochondrial classes to differ in age-related changes in redox status, it was of interest to learn whether that resulted from differential accumulation of mitochondrial mutations, which might be caused by pool differences. Not shown is our finding that the mitochondrial dNTP pools in tissues of aged rats do not differ appreciably from those of the young rats whose values are shown here. Thus, if there is an age-related increase in the mitochondrial genome mutation rate, it results from factors other than dNTP pool changes.

In collaboration with Drs. William Copeland and Thomas Kunkel we obtained evidence suggesting that the pool asymmetries recorded above, especially in heart and skeletal muscle, strongly influence both the mutation rate and the kinds of mutations observed (10). Dr. Zac Pursell in the Kunkel lab carried out gap-filling reactions catalyzed by recombinant human DNA polymerase γ, under conditions where replication errors in a *lacZ* mutational target could be scored as change in plaque color resulting from use of an M13 phagemid vector as the template. Reactions were run at dNTP concentrations mimicking our estimated intramitochondrial values, and with both wild-type polymerase and a mutant enzyme defective in 3'-exonucleolytic proofreading. Error rates were increased by up to threefold with wild-type polymerase and up to sixfold with the proofreading-defective mutant enzyme, as shown in Table II.

Sequencing a large number of the mutants confirmed that most were substitutions and that the mutation stimulated most dramatically by mitochondrial pool asymmetry was AT→GC transitions, where T in the template strand is followed by C, giving a strong

next-nucleotide effect in the presence of high [dGTP]. This is the class of mutation expected in the presence of high [dGTP} and low [dTTP], respectively. Single-base deletions were also strongly stimulated under these conditons.

Table II. Replication errors induced by dNTP asymmetry in polymerase γ reactions

dNTP concentrations	Mutant frequency x 10^4	
	Wild-type pol γ	3' exo-minus pol γ
1 mM equimolar	11	62
1 μM equimolar	7.8	45
Heart mitochondria, SSM	23	160
Hear mitochondria, IFM	21	170
Liver mitochondria	<22	42
Brain mitochondria	ND	60
Skeletal muscle mitochondria	13	270

We next asked whether the AT→GC transition is favored in the spectrum of natural mitochondrial mutations. In two studies with humans and one study with mice, we found this mutation to predominate, representing from 55 to 62 percent of total mutations seen in the three studies (11, 12, 13). Thus, it appears likely that natural mitochondrial dNTP asymmetry, at least in heart and skeletal muscle, influences the spontaneous mutation rate and spectrum.

The true physiological function of "deoxyribonucleotide carrier protein"

Some time ago we were contacted by Dr. Leslie Biesecker at NIH. His laboratory is studying a condition called Amish lethal microcephaly, or Amish disease, in which mitochondria contain subnormal amounts of DNA, although there seems not to be genomic instability (14). The condition has been traced to deficiency of a protein called deoxyribonucleotide carrier protein, or DNC, originally described in 2001 by Dolce *et al*. (15)). This protein, isolated as a recombinant product and incorporated into liposomes, was reported to facilitate deoxyribonucleotide transport, with diphosphates being the preferred substrates. Biesecker's laboratory had generated knockout mice lacking this protein, and they asked us to measure mitochondrial dNTP pools, to test the presumed physiological role of this enzyme in intramitochondrial nucleotide transport. Although we predicted that the DNC knockout would affect mitochondrial dNTP pools, we saw no significant effect of the mutation, as shown in Table III.

Next the Biesecker laboratory told us that they had two cultured lymphoblast lines from Amish disease patients. They sent these to us, along with lymphoblasts from two normal control individuals. Again, as shown also in Table III, we saw no significant effects of the mutation upon mitochondrial dNTP pools.

Table III. Effects of DNC mutations on mitochondrial nucleoside triphosphate pools

Cells	NTP pool, pmol/mg mitochondrial protein					
	dATP	dTTP	dCTP	dGTP	ATP	GTP
Mouse embryo						
Dnc +/+	3.0	2.9	5.0	12.8	ND	ND
Dnc –/–	3.2	5.5	6.4	13.9	ND	ND
Human lympho-blasts						
Amish disease	4.9	19.5	7.0	18.0	2690	1230
Control	4.4	14.6	5.9	20.2	2830	1270

ND, not determined. CTP and UTP pools were too low in our mitochondrial extracts for detection by the HPLC system that we used. All human lymphoblast data are average values seen with two cell lines each.

At that point I recalled, from the paper of Dolce *et al*, that the DNC protein also was seen to transport ribonucleotides in reconstituted liposomes, with diphosphates again being the preferred substrates. That led us to speculate that the pathological effects of the mutation might result from faulty ribonucleotide transport, rather than deoxyribonucleotide. Therefore, we measured the rNTP levels in extracts of the human cells. Although we could not detect UTP or CTP in our HPLC-based assay with these extracts, the data for ATP and GTP pools showed no significant abnormalities when Amish disease mitochondria were compared with controls.

These data suggest that the physiological function of the DNC protein should be re-evaluated. At that point Dr. Biesecker engaged Dr. Ferdinando Palmieri, an expert on mitochondrial transport systems. From the sequence of the DNC protein and from sequences of known mitochondrial transporters in yeast, Palmieri predicted that the true function of DNC was transport of thiamine pyrophosphate, not deoxyribonucleotides. Mitochondria from Amish disease patients were found to be deficient in thiamine pyrophosphate and to be deficient in activities of pyruvate dehydrogenase and α-ketoglutarate dehydrogenase activities unless thiamine pyrophosphate was added to the reaction mixture. These observations, which have recently been published as a collaboration from our three laboratories (16) suggests that the DNC protein is not involved in deoxyribonucleotide transport, and it reopens the entire question of how mitochondrial dNTP pools come into existence.

Extramitochondrial dNTP pools in nonproliferating cells

Our finding that dGTP comprises as much as 90 percent of the dNTP pool in some mitochondria raises the possibility, however unlikely, that it is serving a function in addition to its role in DNA replication. The literature on non-DNA-related functions of deoxyribonucleotides is very sparse, but we were interested to see a paper by Regnier *et al* (17), which reported that rat cardiac muscle contraction in an *in vitro* preparation was stimulated more effectively by dATP than by ATP. If such a function for dNTPs actually

exists in living animals, then we expect to see substantial extramitochondrial dNTP pools. In a preliminary experiment, we compared mitochondrial to cytosolic dNTP pools in rat heart and liver. As shown in Table IV, we did see substantial cytosolic pools. In liver the cytosolic and mitochondrial pools were about equal, while in heart the cytosolic pools exceeded those in mitochondria by up to ninefold. However, these values are still far less than what we normally see when we measure ribonucleoside triphosphate pools. In both heart and liver dGTP predominated among the cytosolic dNTPs and dTTP was the least abundant, just as seen in mitochondria. This may mean that the cytosolic pools are precursors to the mitochondrial pools. The data do not support the premise that dNTPs are playing significant metabolic roles other than their function as DNA precursors.

Table IV. dNTP distribution between rat tissue mitochondria and cytosol

Cell fraction	dNTP content of tissue sample, pmol			
	dATP	dTTP	dCTP	dGTP
Heart cytosol	49.7	17.4	81.1	824.8
Heart mitochondria	18.0	1.8	42.7	748.1
% in mitochondria	*26.6*	*9.4*	*34.3*	*47.6*
Liver cytosol	126.7	32.7	344.2	398.3
Liver mitochondria	145.4	23.3	337.5	349.2
% in mitochondria	*53.4*	*41.6*	*50.2*	*46.7*

A novel form of ribonucleotide reductase in mitochondria

We have preliminary data suggesting that liver mitochondria contain a form of ribonucleotide reductase that is quite different from known forms of this important enzyme. In experiments with rat, mouse, or pig liver, we find the specific activity of ribonucleotide reductase to be severalfold higher in mitochondrial than in cytosolic extracts. This appears to rule out the possibility that the activity in mitochondria represents a cytosolic contaminant. Moreover, the enzyme in mitochondria appears to be immunologically unrelated to any of the well-studied forms of this enzyme. Because the activity in mitochondrial extracts is low, a desirable goal is to isolate the enzyme as a recombinant protein. Analysis of a library of human expressed sequences indicated the presence of a cDNA very closely related to the gene for the well-known R1 protein (large subunit) of the enzyme. We have amplified a portion of this DNA by reverse transcriptase PCR, beginning with a human mRNA library. A gel band of the expected size and restriction pattern was seen. Very recently we have succeededin cloning this fragment into a standard cloning vector, and we are now awaiting DNA sequence data, which should tell whether we have indeed cloned the gene for the putative novel ribonucleotide reductase.

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